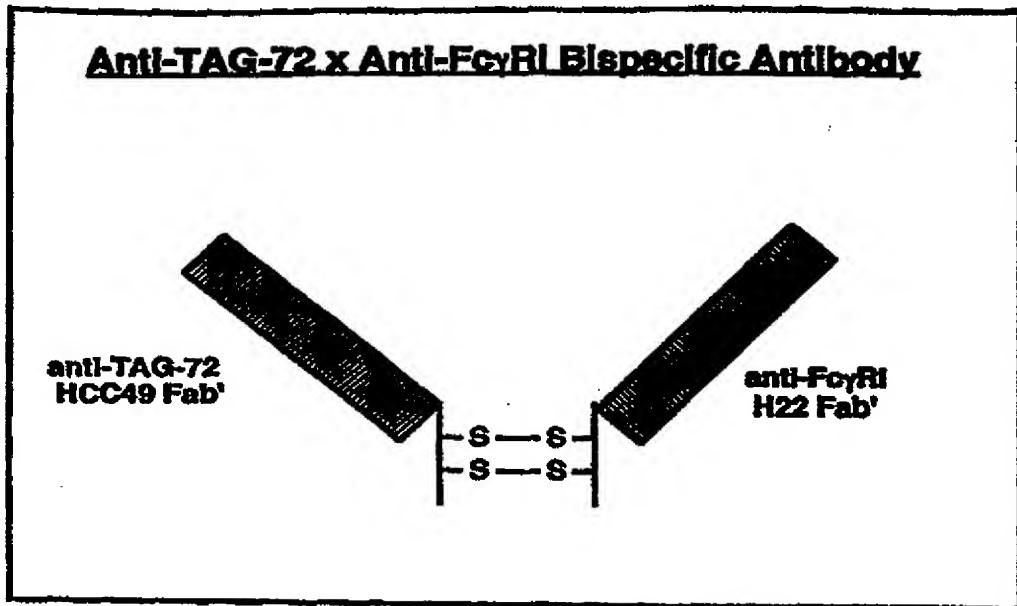




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ :	A1	(11) International Publication Number: WO 99/19362
C07K 16/28, 16/30, 16/46, C12N 5/08, A61K 39/395		(43) International Publication Date: 22 April 1999 (22.04.99)
(21) International Application Number: PCT/US97/18428		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 15 October 1997 (15.10.97)		
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(54) Title: BISPECIFIC MOLECULES DIRECTED TO TUMOR ASSOCIATED GLYCOPROTEIN-72 AND Fc RECEPTOR



(57) Abstract

Bispecific and multispecific molecules which bind to an Fc γ receptor and the tumor associated glycoprotein 72 (TAG-72) are disclosed. The molecules can bind to both an Fc γ receptor-expressing cell and a TAG-72-expressing cell. The specific molecules are useful for targeting human effector cells to TAG-72-expressing cells *in vivo* and *in vitro*. Also disclosed are therapeutic and diagnostic uses of these bispecific molecules.

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**Bispecific Molecules Directed to Tumor Associated
Glycoprotein-72 and Fc Receptors**

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Background of the Invention

Tumor associated glycoprotein (TAG) 72 is a high molecular weight sialylated glycoprotein, which was originally isolated from the human LS174T tumor cell line (Thor, A. *et al.* (1997) *Cancer Res* 46: 3118; Soisson A. P. *et al.* (1989) *Am. J. Obstet. Gynecol.*:1258-63).

10 Expression of TAG-72 has been shown to be associated with a number of adenocarcinomas such as colon, breast, prostate, ovarian and endometrial cancer (Thor, A. *et al.* (1997) *supra*). Unlike the majority of normal adult tissues which do not express detectable amounts of TAG-72, the normal endometrium has been shown to express high levels of TAG-72 (Soisson A. P. *et al.* (1989) *supra*).

15 The restricted expression of TAG-72 on predominantly neoplastic tissues has prompted several groups to explore the utility of TAG-72 as a tumor marker. Numerous murine monoclonal antibodies have been developed which have binding specificity for human TAG-72. One of these monoclonal antibodies, designated B72.3, is a murine IgG1 produced using a human breast carcinoma extract as the immunogen (Colcher, D. 20 *et al.* (1981) *Proc. Natl Acad. Sci. USA* 78: 3199-3203; U.S. Patent Nos: 4,522, 918 and 4,612, 282). Other monoclonal antibodies directed against TAG-72 have been generated. In particular, CC monoclonal antibodies ("CC" refers to colon cancer) were prepared using TAG-72 purified using the B72.3 monoclonal antibody. A number of the CC monoclonal antibodies have been shown to have relatively high binding affinities for 25 human TAG-72, including CC11, CC15, CC30, CC46, CC49, CC83 and CC92 (EP 0365997; Kuroki *et al.* (1990) *Cancer Research* 50:4872-4879). For example, the murine monoclonal CC49 has been shown to react with human ovary, breast and lung tumor tissue, as well as inflammatory colon tissue (Molinolo *et al.* (1990) *Cancer Research* 50:1291-1298). The CC49 antibody has also been shown to localize with high 30 frequency to prostate cancer. The localization of CC49 to prostate cancer has been correlated with anti-tumor effects, specifically with pain relief in individuals afflicted with prostate cancer (Meredith *et al.* (1994) *J. Nuc. Med.* 35:1017-1022).

The applications of murine monoclonal antibodies such as CC49 antibodies to a number of human therapies have been limited by the development of adverse immune 35 responses to the "foreign" murine proteins. These immune response are known as human anti-mouse antibody or HAMA response (Schroff, R. *et al.* (1985), *Cancer Res.*, 45, 879-885). HAMA responses cause serum sickness in humans and result in rapid clearance of the murine antibodies from an individual's circulation. The immune

- 2 -

response in humans has been shown to be against both the variable and the constant regions of murine immunoglobulins.

Recombinant DNA techniques have been used to alter antibodies, for example, by substituting specific immunoglobulin regions from one species with immunoglobulin regions from another species. For example, "chimeric antibodies" have been generated in which the complementary heavy and light chain variable domains of an Ig molecule from one species such as murine, may be combined with the complementary heavy and light chain Ig constant domains from another species, e.g., human (Neuberger *et al.* PCT/GB85/00392). An improvement to these techniques of altering antibodies involves the process of "CDR grafting" or substituting the complementarity determining regions (CDRs) with those from another species as described by Winter *et al.* GB2188538A. This process has been used to substitute the CDRs from the murine variable region domains of a monoclonal antibody with desirable binding properties into human heavy and light chain Ig variable region domains. These altered Ig variable regions have been combined with human Ig constant regions to create antibodies which are totally human in composition except for the substituted murine CDRs. The "reshaped" or "humanized" antibodies described by Winter have been shown to elicit a considerably reduced immune response in humans compared to chimeric antibodies because of the considerably less murine components. In addition, the half life of the altered antibodies in circulation has been shown to approach that of natural human antibodies.

Humanized anti-TAG-72 antibodies have been generated. For example, humanized CC49 monoclonal antibodies (HCC49) which retain the binding specificity for TAG 72 have been described in Slavin-Chiorini *et al.* (1995) *Cancer Research* suppl. 55:5957s-5967s. HCC49 antibodies have been shown to have strong immunoreactivity with human primary prostate cancer and are currently in phase III clinical trials for use as imaging agents (Brenner *et al.* (1995) *Urology* 153:1575-1579).

Summary of the Invention

The present invention features bispecific and multispecific molecules comprising at least one first binding specificity for an Fc receptor and a second binding specificity for a target cell antigen, e.g., a tumor associated antigen present on a target cell. In one embodiment, the invention provides a bispecific molecule, e.g., a bispecific antibody, which binds both to the cytotoxic trigger molecule, human Fc γ RI, and to the human tumor associated glycoprotein 72 (TAG-72). The bispecific and multispecific molecules of the invention can be used as diagnostic or therapeutic agents *in vivo* and *in vitro*.

- 3 -

Multispecific molecules of the invention include trispecific, tetraspecific and other multispecific molecules. In one embodiment the multispecific molecule includes an anti-enhancement factor (EF) portion, e.g., a molecule which binds to a surface protein involved in cytotoxic activity.

5 In preferred embodiments, the bispecific and multispecific molecules of the invention comprise at least one antibody, or a fragment thereof, including, e.g., an Fab, Fab', F(ab')₂, Fv, or a single chain Fv. In particularly preferred embodiments, the antibody or fragment thereof is "humanized" (e.g., has at least a complementarity determining region (CDR) or a portion thereof derived from a non-human antibody (e.g., 10 murine) with the remaining portion(s) are human in origin). In one embodiment, the invention provides a bispecific or multispecific molecule (e.g., antibody) comprising at least two humanized antibodies.

The Fc receptor recognized by the bispecific and multispecific molecules of the invention is preferably a human IgG receptor, e.g., an Fc-gamma receptor (Fc γ R), such 15 as Fc γ RI (CD64), Fc γ RII(CD32), and Fc γ RIII (CD16). In one embodiment, the Fc γ receptor is the high affinity Fc γ receptor, Fc γ RI. The Fc receptor is preferably located on the surface of an effector cell, e.g., a monocyte, macrophage or an activated polymorphonuclear cell. In particularly preferred embodiments, the bispecific and multispecific molecules bind to an Fc receptor at a site which is distinct from the 20 immunoglobulin (e.g., IgG) binding site of the receptor. Therefore, the binding of the bispecific and multispecific molecules is not blocked by physiological levels of immunoglobulins.

In a particular embodiment of the invention, the binding specificity for an Fc receptor, e.g., Fc γ RI, comprises an anti-Fc γ RI antibody, or a fragment thereof. 25 Exemplary anti-Fc γ RI antibodies include mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. In preferred embodiments, a humanized form of the anti-Fc γ receptor antibody is used, such as humanized monoclonal antibody 22 (H22), or a fragment thereof.

The bispecific and multispecific molecules of the invention further comprise a second binding specificity which recognizes, e.g., binds to, a target cell antigen, e.g., a 30 tumor associated antigen. In one embodiment, the tumor associated antigen is the tumor associated glycoprotein-72 (TAG-72), most preferably human TAG-72. Accordingly, the bispecific or multispecific molecule binds to the surface of a TAG-72-expressing cell, e.g., a tumor cell or an endometrial cell. The binding specificity for TAG-72 can be provided by an anti-TAG-72 antibody, or a fragment thereof. For example, the anti- 35 TAG-72 antibody can be a first or a second generation monoclonal anti-TAG-72 antibody, or a fragment thereof. Exemplary anti-TAG-72 antibodies include B72.3,

- 4 -

CC11, CC15, CC30, CC46, CC49, CC83, CC92 and fragments of these antibodies. Among these antibodies, CC49, humanized CC49 (HCC49) and fragments thereof are particularly preferred.

The bispecific and multispecific molecules of the invention can be produced, for example, by recombinant techniques and/or chemical methods. In preferred embodiments, the bispecific and multispecific molecules are produced by chemically conjugating the binding specificities making up the molecules, e.g., by crosslinking an anti-Fc antibody or a fragment thereof, e.g., an Fab' fragment, and an anti-TAG-72 antibody.

In another aspect, the present invention provides a pharmaceutical composition (e.g., for therapeutic or diagnostic use) comprising a bispecific or multispecific molecule comprising at least one binding specificity for an Fc receptor, e.g., an anti-Fc receptor antibody, and a second binding specificity for a tumor associated antigen, e.g., an anti-TAG-72 antibody, and a pharmaceutically acceptable carrier.

In another aspect, the present invention provides a target-specific effector cell which comprises an effector cell expressing an Fc_y receptor, e.g., a macrophage or an activated PMN cell, and a bispecific or multispecific molecule of the invention.

In another aspect, the present invention provides methods of using bispecific and multispecific molecules of the invention to trigger at least one Fc receptor-mediated effector cell activity, such as antibody dependent cellular cytotoxicity (ADCC), phagocytosis of a target cell, e.g., a TAG-72-expressing cell, cytokine release, or generation of superoxide anion, upon binding to the Fc receptor on an effector cell. In one embodiment, the effector cell is contacted with the bispecific or multispecific molecule *in vitro* or *in vivo*. Exemplary effector cells include any Fc-receptor-expressing immune cells such as monocytes, macrophages or activated polymorphonuclear cells. The effector cells can be pretreated with an agent capable of up-regulating Fc-receptor expression, e.g., a cytokine, prior to contacting with a bispecific and multispecific molecule. In a preferred embodiment, the bispecific or multispecific molecule contacts, e.g., binds to, an Fc receptor, and activates, e.g., crosslinks, the Fc receptor. When the Fc receptors are activated a variety of signal transduction events are triggered which result in effector cell activity.

Accordingly, bispecific and multispecific molecules of the invention can be used to induce ADCC and/or phagocytosis of a target cell, e.g., a TAG-72-expressing cell. In one embodiment, the bispecific or multispecific molecule is capable of binding both an Fc receptor expressing effector cell, and a TAG-72-expressing cell. The binding can

- 5 -

occur simultaneously or sequentially. The induction of ADCC and/or phagocytosis can occur *in vivo* or *in vitro*.

Bispecific and multispecific molecules of the invention can also be used to direct an effector cell to a target cell, e.g., a TAG-72-bearing cell either *in vivo* or *in vitro*.

5 Bispecific and multispecific molecules of the invention can also be used in the treatment of, or prophylactic prevention of, a disease characterized by abnormal or aberrant functioning (e.g., activity) of a target cell, e.g., TAG-72-expressing cell, e.g., a TAG-72-expressing tumor cell or endometrial cell, by administering to a subject a pharmaceutical composition of a bispecific or multispecific molecule of the invention, or
10 an effector cell of the invention, in an amount effective to inhibit the abnormal activity of the TAG-72-expressing cells. The subject can be additionally treated with an agent that modulates, e.g., enhances or inhibits, the expression or activity of Fc γ receptors, by for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the bispecific and multispecific molecule include of granulocyte
15 colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), and tumor necrosis factor (TNF).

In yet another aspect, the invention provides methods for detecting *in vitro* and *in vivo* expression of an Fc γ receptor or a TAG-72 protein on an effector cell or a target cell, respectively. The method comprises (i) contacting a sample or a control sample
20 under conditions that allow interaction of a bispecific or multispecific molecule and an Fc γ receptor or TAG-72 protein, and (ii) detecting formation of a complex. A statistically significant change in the formation of the complex with respect to a control sample in the presence of the test compound is indicative of expression of Fc γ receptor, or TAG-72 protein.

25 Other features and advantages of the instant invention will become more apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 is a schematic diagram of a bispecific molecule, HCC49 X H22, having
30 an anti-TAG-72 binding specificity, which is provided by an HCC49 humanized Fab', and an anti-Fc γ RI binding specificity, which is provided by an H22 Fab'.

Figure 2 shows an SDS-PAGE analysis of HCC49 X H22. Lanes 1-4 show samples analyzed in reducing conditions, lanes 6-9 show samples analyzed in non-reducing conditions. Lanes 1 and 9 show the low molecular weight and high molecular
35 weight standards, respectively; lanes 2 and 6 show a sample containing 5 μ g of HCC49

- 6 -

X H22; lanes 3 and 7 show a sample containing 5 µg of H22 F(ab')₂; and, lanes 4 and 8 show a sample containing 5 µg of CC49 F(ab')₂.

Figures 3A-C are schematic representations showing a comparison of binding of HCC49 X H22 to various human carcinoma cell lines as measured by flow cytometry.

- 5 Figures 3A-C show the binding of HCC49 X H22, H22 F(ab')₂ and control to human endometrial carcinoma cells (Kleb cells; Fig. 3A); acute human T cell leukemia cells (Jurkat cells; Fig. 3B) and human colon carcinoma (LS174T; Fig. 3C).

Figures 4A-B are graphic representations of the binding of HCC49 X H22 to various cell lines as measured by flow cytometry. *Figure 4A* shows the binding of HCC49 X H22 (filled circles) and CC49 monoclonal antibody (empty circles) to human endometrial carcinoma cells (Kleb cells). *Figure 4B* shows the binding of HCC49 X H22 to U937 cells.

Figures 5A-B are graphic representations of the simultaneous binding of HCC49 X H22 to various cell lines as measured by bifunctional flow cytometry. *Figure 5A* shows the simultaneous binding of HCC49 X H22 (filled circles) or control anti-EGF X anti-FcγRI (solid circles) to Kleb cells and soluble FcγRI. *Figure 5B* shows the simultaneous binding of HCC49 X H22 to soluble FcγRI and Kleb cells (solid circles), Jurkat cells (empty circles) or CEM cells (filled inverted triangle).

Figures 6A-B are graphic representations showing induction of ADCC of various human carcinoma cell lines using IFNγ-treated monocytes as effector cells. *Figure 6A* shows a comparison of dose response curves demonstrating induction of ADCC of Kleb cells (filled circles) or LS174T cells (empty circles) with respect to the concentration of HCC49 X H22 (µg/ml). *Figure 6B* shows a comparison of dose response curves demonstrating induction of ADCC of Kleb cells (filled circles) or Jurkat cells (empty circles) with respect to the concentration of HCC49 X H22 (µg/ml).

Figure 7 is a dose response curve showing induction of ADCC of TAG-72 overexpressing Kleb cells using IFNγ and G-CSF pretreated PMNs as effector cells with respect to the concentration of HCC49 X H22 (filled circles) or CC49 monoclonal antibody (empty circles).

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Detailed Description of the Invention

The present invention is based in part on the discovery that effector cells expressing Fc receptors can be successfully targeted via their Fc receptors to particular tumor antigen-bearing cells to promote death of the target cells. More particularly, as part of this invention, it was discovered that targeted cytotoxicity of cells bearing the tumor associated glycoprotein 72 (TAG-72) could be achieved using bispecific and

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multispecific molecules comprising at least one binding specificity for an Fc receptor located on the surface of an effector cell and a second binding specificity for a TAG-72 antigen. By physically bringing together cytotoxic effector cells and TAG-72 bearing tumor cells using bispecific or multispecific molecules of the invention, more efficient, 5 targeted elimination of such undesirable tumor cells can be achieved.

Accordingly, in one embodiment, the invention provides a bispecific or multispecific molecule comprising a first binding specificity for the cytotoxic trigger molecule, Fc γ R1, and a second binding specificity for the tumor associated glycoprotein 72 (TAG-72), preferably human TAG-72. These bispecific and multispecific molecules 10 are capable of binding both to Fc γ R1-expressing effector cells (e.g., monocytes, macrophages or polymorphonuclear cells (PMNs)), and to target cells expressing TAG-72, e.g., a carcinoma-derived tumor cell, or an endometrial cell. When binding in this manner, the bispecific and multispecific molecules trigger Fc receptor-mediated effector cell activities, such as antibody dependent cellular toxicity (ADCC), phagocytosis of a 15 target cell, cytokine release, or generation of superoxide anion.

The term "bispecific molecule" is intended to include any agent, e.g., a protein, peptide, or protein or peptide complex, which has two different binding specificities which bind to, or interact with (a) a cell surface antigen and (b) an Fc receptor on the surface of an effector cell. The term "multispecific molecule" or "heterospecific 20 molecule" is intended to include any agent, e.g., a protein, peptide, or protein or peptide complex, which has more than two different binding specificities which bind to, or interact with (a) a cell surface antigen, (b) an Fc receptor on the surface of an effector cell, and (c) at least one other component. Accordingly, the invention includes, but is not limited to, bispecific, trispecific, tetraspecific, and other multispecific molecules 25 which are directed to cell surface antigens, such as TAG-72, and to Fc receptors on effector cells.

As used herein, the term "heteroantibodies" refers to two or more antibodies, antibody binding fragments (e.g., Fab), derivatives therefrom, or antigen binding regions linked together, at least two of which have different specificities. These different 30 specificities include a binding specificity for an Fc receptor on an effector cell, and a binding specificity for an antigen or epitope on a target cell.

Multispecific molecules of the invention can further include a third binding specificity, in addition to an anti-Fc binding specificity and an anti-target cell antigen binding specificity, such as TAG-72. In one embodiment, the third binding specificity is 35 an anti-enhancement factor (EF) portion, e.g., a molecule which binds to a surface protein involved in cytotoxic activity and thereby increases the immune response against

the target cell. The "anti-enhancement factor portion" can be an antibody, functional antibody fragment or a ligand that binds to a given molecule, e.g., an antigen or a receptor, and thereby results in an enhancement of the effect of the binding determinants for the F_C receptor or target cell antigen. The "anti-enhancement factor portion" can

5 bind an F_C receptor or a target cell antigen. Alternatively, the anti-enhancement factor portion can bind to an entity that is different from the entity to which the first and second binding specificities bind. For example, the anti-enhancement factor portion can bind a cytotoxic T-cell (e.g. via CD2, CD3, CD8, CD28, CD4, CD40, ICAM-1 or other immune cell that results in an increased immune response against the target cell).

10 In one embodiment, the bispecific and multispecific molecules of the invention comprise as a binding specificity at least one antibody, or an antibody fragment thereof, including, e.g., an Fab, Fab', F(ab')₂, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv or a single chain construct as described in Ladner et al. U.S. Patent No. 4,946,778, issued
15 August 7, 1990, the contents of which is expressly incorporated by reference. The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. In another embodiment of the invention, the bispecific and multispecific
20 molecule comprises a chimeric antibody or binding fragment thereof. A "chimeric antibody" is intended to include an antibody in which the variable regions are from one species of animal and the constant regions are from another species of animal. For example, a chimeric antibody can be an antibody having variable regions which derive from a mouse monoclonal antibody and constant regions which are human. In a
25 preferred embodiment of the invention, the bispecific and multispecific molecule comprises a humanized antibody or binding fragment thereof. The term "humanized antibody" is intended to include antibodies in which the hypervariable regions, also termed, the complementarity-determining regions (CDRs) are from one species of animal and the framework regions and constant regions of the antibody are from a
30 different species animal species. In a humanized antibody of the invention, the CDRs are from a mouse monoclonal antibody and the other regions of the antibody are human. In preferred embodiments, a human antibody is derived from known proteins NEWM and KOL for heavy chain variable regions (VHs) and REI for Ig kappa chain, variable regions (VKs). The term antibody as used herein is intended to include chimeric and
35 humanized antibodies, binding fragments of antibodies or modified versions of such.

- 9 -

- The terms "fragment" or "binding fragment" of an antibody or protein capable of binding to an antigen is intended to include a fragment of the antibody or protein which is sufficient for binding to the antigen. Binding of a binding fragment of an antibody to an antigen can be with the same affinity or a different affinity, e.g., lower or higher
- 5 affinity, as binding of the whole antibody to the antigen. Examples of binding fragments encompassed within the term antibody include: a Fab fragment consisting of the V_L, V_H, C_L and C_{H1} domains; an Fd fragment consisting of the V_H and C_{H1} domains; an Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward et al., 1989 *Nature* 341:544-546) consisting of a V_H
- 10 domain; an isolated complementarity determining region (CDR); and an F(ab')₂ fragment, a bivalent fragment comprising two Fab' fragments linked by a disulfide bridge at the hinge region. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.
- 15 A binding fragment, e.g., a binding fragment of an antibody or of a ligand, can be an active or functional binding fragment. Accordingly, an active or functional binding fragment is intended to include binding fragments which are capable of triggering at least one activity or function triggered by the full length molecule. For example, an active binding fragment of monoclonal antibody M22 or H22 is a fragment
- 20 of the antibody that is capable of binding to the Fc_yR and triggering a receptor mediated effector cell activity, e.g., production of superoxide anion.
- The term "binding specificity" is used interchangeably herein with the terms "antigen binding site", "antigen binding region" and "binding determinant of an antibody". These terms are intended to include the region of a molecule, e.g., an
- 25 antibody, that are involved in the binding to an antigen. The antigen binding site of an antibody comprises, but is not limited to, the amino acids of the antibody which contact the antigen. The antigen binding region can be the variable region of an antibody. The antigen binding region of an antibody can also be the hypervariable regions of an antibody. The antigen binding region of an antibody can also be the amino acid residues
- 30 in the hypervariable region of an antibody which contact the antigen and/or which provide proper tertiary structure of the antigen binding region. Various methods are available for determining which amino acid residues of a variable region or hyper variable region of an antibody contact the antigen and/or are important in having a correctly folded antigen binding region. For example, mutagenesis analyses can be
- 35 performed. In particular, it is possible to substitute one or more amino acids for other amino acids in a recombinantly produced antibody and to perform *in vitro* binding

- 10 -

studies to determine the extent to which the binding affinity of the modified antibody for the antigen has changed compared to the non modified antibody. If binding has decreased due to substitution of an amino acid for another, the amino acid is most likely important in binding of the antibody to the antigen. Other methods for determining
5 which amino acids of a variable region of an antibody are involved in binding of the antibody to an antigen are based on crystallographic analyses, e.g., X-ray crystallography.

The term "an antibody which binds specifically to an antigen" is intended to include an antibody which binds to the specific antigen with significantly higher affinity
10 than binding to any other antigen, i.e., it is intended to define the specificity of an antibody as defined in the art. The terms "an antibody recognizing an antigen" and " an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

Preferred bispecific and multispecific molecules of the invention comprise a
15 binding specificity for an Fc γ R present on the surface of an effector cell, most preferably Fc γ RI, and a second binding specificity for a target cell antigen, e.g., a TAG-72 cell antigen.

In one embodiment, the binding specificity for an Fc receptor is provided by a
20 human monoclonal antibody, the binding of which is not blocked by human immunoglobulin G (IgG). As used herein, the term "IgG receptor" refers to any of the eight γ -chain genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fc γ receptor classes: Fc γ RI (CD64), Fc γ RII(CD32), and Fc γ RIII (CD16). In one preferred embodiment, the Fc γ receptor a human high affinity Fc γ RI. The human Fc γ RI is a 72
25 kDa molecule, which shows high affinity for monomeric IgG (10^8 - 10^9 M $^{-1}$).

The production and characterization of these preferred monoclonal antibodies are described by Fanger et al. in PCT application WO 88/00052 and in U.S. Patent No. 4,954,617, the teachings of which are fully incorporated by reference herein. These antibodies bind to an epitope of Fc γ RI, Fc γ RII or Fc γ RIII at a site which is distinct from
30 the Fc γ binding site of the receptor and, thus, their binding is not blocked substantially by physiological levels of IgG. Specific anti-Fc γ RI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. The hybridoma producing mAb 32 is available from the American Type Culture Collection, ATCC Accession No. HB9469. Anti-Fc γ RI mAb 22, F(ab') $_2$ fragments of mAb 22, and can be obtained from
35 Medarex, Inc. (Annandale, N.J.). In other embodiments, the anti-Fc γ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and

- 11 -

characterization of the H22 antibody is described in Graziano, R.F. et al. (1995) *J. Immunol.* 155 (10): 4996-5002 and PCT/US93/10384. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession no. CRL 11177.

5 In other embodiments, bispecific and multispecific molecules of the invention further comprise a binding specificity which recognizes, e.g., binds to, a target cell antigen, e.g., a tumor associated antigen. In a preferred embodiment, the tumor associated antigen is tumor associated glycoprotein-72 (TAG-72), most preferably human TAG-72. TAG 72 has been identified on about 90% of colorectal cancers, 85%
10 of breast tumors, and 95% of ovarian tumors (Johnson et al. (1986) *Cancer Res.* 46:850-897; Bodmer, M. et al., European Patent Specification 0 348 442 B1; Mezes, P. et al. International Application WO 93/12231). As used herein, the term "TAG-72" refers to a sialylated glycoprotein which has been identified as a mucin characterized by: (a) a high molecular weight ($>1 \times 10^6$); (b) a density of approximately 1.45 gm/ml as determined
15 by equilibrium centrifugation in CsCl; and (c) a change in migration after neuraminidase digestion (PCT/US88/01941).

The binding specificity for TAG-72 can be provided by an anti-TAG-72 antibody, or a fragment thereof. In certain embodiments, the anti-TAG-72 can be a first or a second generation monoclonal anti-TAG-72 antibody, or a fragment thereof. As
20 used herein, a "first generation monoclonal antibody" refers to a monoclonal antibody produced using as immunogen a crude cell extract, for example, B72.3 (PCT/US88/01941). A "second generation monoclonal antibody" refers to a monoclonal antibody produced using an antigen which has been affinity purified with a first generation monoclonal antibody. Exemplary second generation anti-TAG-72 antibodies
25 suitable for use in the invention include CC11, CC15, CC30, CC46, CC49, CC83 and CC92. In preferred embodiments, the anti-TAG-72 antibody is CC49, produced by a hybridoma deposited with the American Type Culture Collection (Rockville, MD) at accession number HB9459. Procedures for generating monoclonal and chimeric forms of CC49 are described in published PCT application WO 93/11161, published PCT
30 application WO 90/04410, corresponding to granted EP Patent No. 365 997; published PCT application WO 93/12231; and published PCT application WO 89/01783. In a preferred embodiment of the invention, a humanized form of CC49 is used, referred to as HCC49, or a fragment thereof (Slavin-Chiorini et al. (1995) *Cancer Research* suppl. 55:5957s-5967s).

35 As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and

- 12 -

activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, e.g., lymphocytes (e.g., B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, macrophages, monocytes, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and
5 basophils. Effector cells express specific Fc receptors and carry out specific immune functions. In preferred embodiments, an effector cell is capable of inducing antibody-dependent cellular toxicity (ADCC), e.g., a neutrophil capable of inducing ADCC. For example, monocytes, macrophages, neutrophils, eosinophils, and lymphocytes which express Fc α R are involved in specific killing of target cells and presenting antigens to
10 other components of the immune system, or binding to cells that present antigens. In other embodiments, an effector cell can phagocytose a target antigen, target cell, or microorganism. The expression of a particular FcR on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of Fc γ RI has been found to be up-regulated by interferon gamma (IFN- γ). This enhanced expression increases the
15 cytotoxic activity of Fc γ RI-bearing cells against targets. An effector cell can phagocytose a target antigen or a target cell. An effector cell can also lyse a target cell.

An "effector cell specific antibody" as used herein refers to an antibody or functional antibody fragment that binds the Fc receptor of effector cells. Preferred antibodies for use in the subject invention bind the Fc receptor of effector cells at a site
20 which is not bound by endogenous immunoglobulin.

"Target cell" shall mean any undesirable cell in a subject (e.g., a human or animal) that can be targeted by a bispecific and multispecific molecule of the invention. In preferred embodiments, the target cell is a TAG-72-bearing cell. Exemplary TAG-72-bearing cells include tumor cells, such as carcinoma or adenocarcinoma-derived cells
25 (e.g., colon, breast, prostate, ovarian and endometrial cancer cells) (Thor, A. *et al.* (1997) *Cancer Res* 46: 3118; Soisson A. P. *et al.* (1989) *Am. J. Obstet. Gynecol.*:1258-63). The TAG-72-bearing cell can also be a normal endometrial cell. The term "carcinoma" is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas,
30 genitourinary system carcinomas, testicular carcinomas, breast carcinomas, ovarian carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues.
35 An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which

- 13 -

the tumor cells form recognizable glandular structures. The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

Production of Antibodies as Binding Specificities

5 A monoclonal anti-Fc (e.g., anti-Fc γ) receptor antibody of this invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology e.g., the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be
10 employed e.g., viral or oncogenic transformation of B lymphocytes.

The preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a very well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also
15 known.

Human monoclonal antibodies (mAbs) directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with
20 specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. International Application WO 92/03918; Kay et al. International Application 92/03917; Lonberg, N. et al. 1994 *Nature* 368:856-859; Green, L.L. et al. 1994 *Nature Genet.* 7:13-21; Morrison, S.L. et al. 1994 *Proc. Natl. Acad. Sci. USA* 81:6851-6855;
25 Bruggeman et al. 1993 *Year Immunol.* 7:33-40; Tuailon et al. 1993 *PNAS* 90:3720-3724; Bruggeman et al. 1991 *Eur J Immunol.* 21:1323-1326).

Monoclonal antibodies can also be generated by other methods known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate
30 antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display see e.g., Sastry et al. 1989 *PNAS* 86:5728; Huse et al. 1989 *Science* 246:1275; and Orlandi et al. 1989 *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are
35 generally known for obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and

- 14 -

PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al., 1991,
5 *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human heavy and light chain variable regions from human antibodies (Larrick et al., 1991, *Methods: Companion to Methods in Enzymology* 2:106-110).

In an illustrative embodiment, RNA is isolated from B lymphocytes, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard
10 protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy
15 and light chains are amplified, each alone or in combinantion, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the
20 amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display
25 libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAPTM* phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for
30 use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al.
35 International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al.

- 15 -

- (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFv gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible (Gly₄-Ser)₃ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFv antibodies immunoreactive with the antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with the Fc γ R, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the Fc γ R. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

Specific bispecific and multispecific molecules with high affinities for a surface protein can be made according to methods known to those in the art, e.g., methods involving screening of libraries (Ladner, R.C., et al., U.S. Patent 5,233,409; Ladner, R.C., et al., U.S. Patent 5,403,484). Further, the methods of these libraries can be used in screens to obtain binding determinants that are mimetics of the structural determinants of antibodies.

In particular, the Fv binding surface of a particular antibody molecule interacts with its target ligand according to principles of protein-protein interactions, hence sequence data for V_H and V_L (the latter of which may be of the κ or λ chain type) is the basis for protein engineering techniques known to those with skill in the art. Details of the protein surface that comprises the binding determinants can be obtained from antibody sequence information, by a modeling procedure using previously determined three-dimensional structures from other antibodies obtained from NMR studies or crystallographic data. See for example Bajorath, J. and S. Sheriff, 1996, *Proteins: Struct., Funct., and Genet.* 24 (2), 152-157; Webster, D.M. and A. R. Rees, 1995,

- 16 -

"Molecular modeling of antibody-combining sites," in S. Paul, Ed., *Methods in Molecular Biol.* 51, Antibody Engineering Protocols, Humana Press, Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: A program to screen aligned nucleotide and amino acid sequences," in *Methods in Molecular Biol.* 51, op.

5 *cit.*, pp 1-15.

Bispecific and multispecific molecules of the invention include those comprising an antigen binding site that is derived from an antibody and which is grafted onto a molecule which is not an antibody. For example, an antigen binding region can be grafted onto a peptide or protein. In a particular embodiment, one portion of the antigen
10 binding region, e.g., the portion similar to the antigen binding region from the light chain of an antibody, is grafted onto one protein or peptide and the other portion of the antigen binding region, e.g., the portion similar to the antigen binding region from the heavy chain of an antibody, is grafted onto another protein or peptide. In a preferred embodiment of the invention, the two proteins or peptides having each a portion of the
15 antigen binding region are linked, e.g., by chemical linkage, recombinantly, or by non covalent interaction, such as to produce a protein having an antigen binding site specific for an Fc γ R for human IgG, which triggers at least one Fc receptor-mediated effector cell function.

An antigen binding region can also be obtained by screening various types of
20 combinatorial libraries with a desired binding activity, and to identify the active species, by methods that have been described. For example, phage display techniques (Marks et al. (1992) *J Biol Chem* 267:16007-16010) can be used to identify proteins binding Fc γ Rs. Phage display libraries have been described above.

In one embodiment, a variegated peptide library is expressed by a population of
25 display packages to form a peptide display library. Ideally, the display package comprises a system that allows the sampling of very large variegated peptide display libraries, rapid sorting after each affinity separation round, and easy isolation of the peptide-encoding gene from purified display packages. Peptide display libraries can be in, e.g., prokaryotic organisms and viruses, which can be amplified quickly, are
30 relatively easy to manipulate, and which allows the creation of large number of clones. Preferred display packages include, for example, vegetative bacterial cells, bacterial spores, and most preferably, bacterial viruses (especially DNA viruses). However, the present invention also contemplates the use of eukaryotic cells, including yeast and their spores, as potential display packages. Phage display libraries are described above.

35 Other techniques include affinity chromatography with an appropriate "receptor", e.g., Fc γ R, to isolate binding agents, followed by identification of the

isolated binding agents or ligands by conventional techniques (e.g., mass spectrometry and NMR). Preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, or luminescent compounds) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor.

Combinatorial libraries of compounds can also be synthesized with "tags" to encode the identity of each member of the library (see, e.g., W.C. Still *et al.*, International Application WO 94/08051). In general, this method features the use of inert but readily detectable tags, that are attached to the solid support or to the compounds. When an active compound is detected, the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels among to total set of all compounds in the library.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (see Robinson *et al.*, International Patent Publication PCT/US86/02269; Akira, *et al.*, European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.*, European Patent Application 173,494; Neuberger *et al.*, International Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent Application 125,023; Better *et al.* (1988 *Science* 240:1041-1043); Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.*, 1987, *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.*, 1987, *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.*, 1988, *J. Natl Cancer Inst.* 80:1553-1559).

The chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General reviews of humanized chimeric antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207 and by Oi *et al.*, 1986, *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from 7E3, an anti-GPII_bIII_a antibody producing hybridoma. The recombinant DNA encoding the

- 18 -

chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution U.S. Patent 5,225,539; Jones et al. 1986 *Nature* 321:552-525; Verhoeyan et al. 1988 *Science* 239:1534; and Beidler et al. 1988 *J. Immunol.* 141:4053-4060.

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor.

An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on March 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as described in International Application WO 94/10332 entitled, *Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear Phagocytes*.

Also within the scope of the invention are chimeric and humanized antibodies in which specific amino acids have been substituted, deleted or added. In particular, preferred humanized antibodies have amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, in a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such substitutions are known to improve binding of humanized antibodies to the antigen in some instances. Antibodies in which amino acids have been added, deleted, or substituted are referred to herein as modified antibodies or altered antibodies.

The term modified antibody is also intended to include antibodies, such as monoclonal antibodies, chimeric antibodies, and humanized antibodies which have been modified by, e.g., deleting, adding, or substituting portions of the antibody. For example, an antibody can be modified by deleting the constant region and replacing it with a constant region meant to increase half-life, e.g., serum half-life, stability or affinity of the antibody. Any modification is within the scope of the invention so long as the bispecific and multispecific molecule has at least one antigen binding region specific for an Fc γ R and triggers at least one effector function.

Methods for Making Bispecific and Multispecific Molecules

The bispecific and multispecific molecules of the present invention can be made using chemical techniques (see e.g., D. M. Kranz et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:5807), "polydoma" techniques (See U.S. Patent 4,474,893, to Reading), or recombinant DNA techniques.

In particular, bispecific and multispecific molecules of the present invention can be prepared by conjugating the constituent binding specificities, e.g., the anti-FcR and anti-target binding specificities, using methods known in the art and described in the examples provided herein. For example, each binding specificity of the bispecific and multispecific molecule can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky et al. (1984) *J. Exp. Med.* 160:1686; Liu, MA et al. (1985) *Proc. Natl. Acad. Sci. USA* 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan et al. (*Science* (1985) 229:81-83), and Glennie et al. (*J. Immunol.* (1987) 139: 2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

When the binding specificities are antibodies (e.g., two humanized antibodies), they can be conjugated via sulphhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulphhydryl residues, preferably one, prior to conjugation.

Alternatively, both binding specificities can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the bispecific and multispecific molecule is a mAb x mAb, mAb x Fab, Fab x F(ab')₂ or ligand x Fab fusion protein. A bispecific and multispecific molecule of the invention, e.g., a bispecific molecule can be a single chain molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Bispecific and multispecific molecules can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- and multispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175;

- 20 -

U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Binding of the bispecific and multispecific molecules to their specific targets can
5 be confirmed by enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), or a Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (e.g., an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using e.g., an enzyme-linked antibody or antibody fragment
10 which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March,
15 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

Pharmaceutical Compositions

20 In another aspect, the present invention provides pharmaceutically acceptable compositions which comprise a bispecific or multispecific molecule of the invention (e.g., which contains at least one binding specificity for an Fc receptor and at least one binding specificity for a tumor associated antigen), formulated together with a pharmaceutically acceptable carrier.

25 In one embodiment, the pharmaceutical composition is administered by combination therapy, i.e., combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one anti-cancer agent, one vaccine, or other conventional therapy. Exemplary anti-cancer agents include cis-platin, adriamycin, and taxol.

30 As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (e.g., by injection or infusion). Depending on the route of
35 administration, the active compound, i.e., bispecific and multispecific molecule, may be

- 21 -

coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired

- 5 toxicological effects (see e.g., Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-
10 substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine
15 and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid

- 20 release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g.,
25 *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to
30 a subject in an appropriate carrier, for example, liposomes, or a diluent.

Pharmaceutically acceptable diluents include saline and aqueous buffer solutions.

Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27). Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the
35 extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except

- 22 -

insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the
5 conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can
10 be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.
Prolonged absorption of the injectable compositions can be brought about by including
15 in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration.
20 Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional
25 desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially
30 advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The
35 specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular

- 23 -

therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium

5 metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

10 For the therapeutic compositions, formulations of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

15 Formulations of the present invention which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate. Dosage forms for the topical or transdermal administration of compositions of this invention 20 include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

25 The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, 30 subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

- 24 -

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (more preferably, 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

- 25 -

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required
5 in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be intravenous,
10 intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic compositions may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is
15 preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413,
20 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent
25 No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein
30 by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes.
35 For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548;

- 26 -

and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (see, e.g., U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); see also K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273. In one embodiment of the invention, the therapeutic compounds of the invention are formulated in liposomes; in a more preferred embodiment, the liposomes include a targeting moiety. In a most preferred embodiment, the therapeutic compounds in the liposomes are delivered by bolus injection to a site proximal to the tumor or infection. The composition must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi.

A "therapeutically effective dosage" preferably inhibits tumor growth by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit cancer can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease tumor size, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol

- 27 -

or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

- When the active compound is suitably protected, as described above, the
5 compound may be orally administered, for example, with an inert diluent or an assimilable edible carrier.

Uses and Methods of the Invention

The multispecific and bispecific molecules of the present invention have *in vitro*
10 and *in vivo* diagnostic and therapeutic utilities. For example, these molecules can be administered to cells in culture, e.g. *in vitro* or *ex vivo*, or in a subject, e.g., *in vivo*, to treat or diagnose a variety of disorders. As used herein, the term "subject" is intended to include human and non-human animals. Preferred human animals include a human patient having a disorder characterized by abnormal functioning of a target cell, e.g., a
15 TAG-72-expressing cell. The term "non-human animals" of the invention includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

Multispecific or bispecific molecules of the invention can be initially tested for binding activity associated with therapeutic or diagnostic use *in vitro*. For example, the
20 activity of these molecules in triggering at least one Fc receptor-mediated effector cell activity, such as antibody dependent cellular cytotoxicity (ADCC), phagocytosis of a target cell, e.g., a TAG-72-expressing cell, cytokine release, or generation of superoxide anion can be assayed. Protocols for assaying *in vitro* activity of multispecific or bispecific molecules in inducing superoxide induction are described in Graziano *et al.*
25 (1995) *J. Immunol.* 155: 4996-5002. Protocols for assaying induction of ADCC *in vitro* are described in the Example below and in Graziano *et al.* (1995) *J. Immunol.* 155: 4996-5002 and in Keler, T. *et al.* (1997) *Cancer Research* 57:4008-14). Protocols for detecting changes in cytokine concentration can be detected via a variety of immunoassays, such as enzyme-linked immunoassay (ELISA), enzyme immunoassay
30 (EIA) or radioimmunoassay (RIA) which are known in the art (see e.g., Keler, T. *et al.* (1997) *supra*). Exemplary cytokines that can be assayed include: granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), interleukins 1 -12 (IL-1 to IL-12), and TNF- α .

35 Preferred bi- or multispecific molecules of the invention stimulate phagocytosis of target cells by effector cells when the molecules links the target cell to the effector

cell. Phagocytosis assays can be performed as follows. Packed target cells can be mixed for 16 hours at 10° C with 20 µl of the F(ab')₂-Ig conjugates at concentrations previously determined to give maximal rosette formation. Heteroantibody-coated OE are washed, adjusted to 4 x 10⁷ cells/ml, and are mixed with an equal volume of 5 myeloid cells at 2 x 10⁶ cells/ml. This mixture is incubated for 10 min at 37° C, and the cells are pelleted and incubated for a further 20 min, after which time noningested OE are lysed at 4° C with buffered ammonium chloride. Phagocytosis can be assessed by microscopic examination of Wright's Giemsa (Sigma) stained cytocentrifuge preparations. At least 200 cells are counted in duplicate slides. Phagocytosis can be quantified as the 10 percentage of cells containing one or more ingested erythrocyte(s).

The bispecific or multispecific molecules of the invention have additional utility in therapy and diagnosis. For example, the bispecific or multispecific molecules can be used as a targeting agent e.g., to target cells expressing an Fcγ receptor to a TAG-72-expressing cell. For example, the bispecific or multispecific molecules can be used to 15 target lipid vesicles containing drugs, e.g., anticancer drugs, for treatment of certain diseases, e.g., cancers (e.g. carcinoma or adenocarcinoma-derived) or endometriosis, or to target lipid vesicles containing factors (such as gamma-IFN) to, for example, activate effector cells. The multi- or bispecific molecules can be used directly *in vivo* to eliminate TAG-72-expressing cells via natural complement or ADCC mechanisms.

For example, the bispecific or multispecific molecules of the invention can be used in the treatment of sarcomas, carcinomas and/or leukemias. Exemplary disorders for which the subject method may be used as part of a treatment regimen include: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endothelioma, lymphangiosarcoma, 20 lymphangioendothelioma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, 25 bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendrogioma, 30 meningioma, melanoma, neuroblastoma, and retinoblastoma.

- 29 -

In addition, the bispecific or multispecific molecules of the invention can be used to treat such disorders as carcinomas forming from tissue of the breast, prostate, kidney, bladder or colon.

Methods of administering bispecific or multispecific molecules of the invention
5 are known in the art. Suitable dosages of the molecules used will depend on the age and weight of the subject and the particular drug used. The molecules can be coupled to radionuclides, such as 131I, 90Y, 105Rh, etc., as described in Goldenberg, D.M. *et al.* (1981) *Cancer Res.* 41: 4354-4360, and in EP 0365 997. The bispecific or multispecific molecules of the invention can also be coupled to anticancer drugs, including one or
10 more agents selected from the group consisting of mitotic inhibitors, alkylating agents, antimetabolites, nucleic acid intercalating agents, topoisomerase inhibitors, toxins, agents which promote apoptosis, and agents which increase immune responses to tumors.

Targeted effector cells, e.g., effector cells linked to bispecific or multispecific
15 molecules of the invention can also be used as therapeutic agents. Effector cells for targeting can be human leukocytes such as macrophages, neutrophils or monocytes. Other cells include and other IgG-receptor bearing cells. If desired, effector cells can be obtained from the subject to be treated. The targeted effector cells, can be administered as a suspension of cells in a physiologically acceptable solution. The number of cells
20 administered can be in the order of 10^8 - 10^9 but will vary depending on the therapeutic purpose. In general, the amount will be sufficient to obtain localization at the target cell and to effect target cell killing by antibody dependent-mediated cytotoxicity (ADCC). Routes of administration can also vary. In tumor therapy, for instance, depending upon the localization of a tumor, the targeted effector cells could be administered
25 intravenously, or directly into tumor sites; as for example, directly into the peritoneal cavity in the case of ovarian carcinoma.

Therapy with targeted effector cells can be performed in conjunction with other techniques for removal of targeted cells. For example, anti-tumor therapy using bispecific or multispecific molecules of the invention and/or effector cells armed with
30 bispecific or multispecific molecules of the invention can be used in conjunction with surgery, chemotherapy or radio-therapy. Additionally, combination immunotherapy may be used to direct two distinct cytotoxic effector populations toward tumor cell rejection. For example, anti-tumor antibodies linked to anti-Fc-gammaRI or anti-T3 (will trigger cytolytic T lymphocytes to lyse tumor cells) may be used in conjunction
35 with IgG-receptor specific binding agents, e.g., heteroantibodies. Protocols based on

- 30 -

these concepts may be especially effective in removing residual tumor cells in patients induced into remission by chemotherapy and irradiation.

Bispecific and multispecific molecules of the invention can also be used to modulate Fc γ R levels on effector cells, such as by capping and elimination of receptors 5 on the cell surface. Mixtures of anti-Fc receptors can also be used for this purpose.

Bispecific and multispecific molecules of the invention which have complement binding sites, such as portions from IgG1, -2, or -3 or IgM which bind complement can also be used in the presence of complement. In one embodiment, *ex vivo* treatment of a population of cells comprising target cells with a binding agent of the invention and 10 appropriate effector cells can be supplemented by the addition of complement or serum containing complement. Phagocytosis of target cells coated with a binding agent of the invention can be improved by binding of complement proteins. In another embodiment target cells coated with the multi- or bispecific molecules of the invention can also be lysed by complement.
15 Bispecific and multispecific molecules of the invention used *in vitro* or *in vivo* can also be administered together with complement. Accordingly, within the scope of the invention are compositions comprising multi- or bispecific molecules and serum or complement. These compositions are advantageous in that the complement is located in close proximity to the bispecific or multispecific molecule. Alternatively, the bispecific 20 or multispecific molecules of the invention and the complement or serum can be administered separately.

Also within the scope of the invention are kits comprising bispecific or multispecific molecules of the invention and instructions for use. The kit can further contain at least one additional reagent, such as complement, or one or more additional 25 bispecific or multispecific molecules of the invention.

Further within the scope of the invention are methods for treating a disorder in a subject characterized by abnormal functioning of a TAG-72-expressing cell, such as a cancer, or endometriosis using bispecific or multispecific molecules of the invention, alone or in combination. In one embodiment, the invention provides methods for 30 treating *in vivo* human carcinomas or endometriosis. A combination of bispecific or multispecific molecules of the invention can be used for this purpose, e.g., a combination of a first binding agent having at least one antigen binding region specific for an Fc γ R and at least one antigen binding region to TAG-72, and a second binding agent having an antigen binding region to a different epitope of TAG-72 and/or Fc γ R.
35 In certain embodiments, a second bispecific or multispecific molecules of the invention can be used in conjunction with the first. For example, this second bispecific or

- 31 -

multispecific molecule can have at least one antigen binding region specific for an IgA receptor, e.g., Fc α receptor, IgE receptor, e.g., Fc ϵ receptor, an Fc δ receptor and/or an Fc μ receptor. Determination of therapeutically affective amounts and dose regimen of the multi- or bispecific molecule can be performed by the skilled artisan using the assays
5 described in the art.

In other embodiments, the subject can be additionally treated with an agent that modulates, e.g., enhances or inhibits, the expression or activity of Fc γ receptors, by for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the multispecific molecule include of granulocyte colony-
10 stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN- γ), and tumor necrosis factor (TNF).

Bispecific and multispecific molecules of the invention can also be used to target cells expressing Fc γ R or TAG-72, for example for labeling such cells. For such use, the binding agent can be linked to a molecule that can be detected. Thus, the invention
15 provides methods for localizing *ex vivo* or *in vitro* cells expressing Fc γ R or TAG-72. The detectable label can be, e.g., a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. In one embodiment, the invention provides methods for diagnosing *in vitro* and *in vivo* human carcinomas, comprising (i) obtaining a body sample, such as a body fluid, tissue or biopsy from a patient; (ii) contacting the body
20 sample with a multi- or bispecific molecule of the invention or a fragment thereof; (iii) determining the level of binding of said multi- or bispecific molecule to the body sample; (iv) comparing the amount of molecule bound to the body sample to a control sample or to a predetermined base level, so that a binding greater than the control level is indicative of the presence of human carcinoma.

25 In still another embodiment, the invention provides a method for detecting the presence of a TAG-72-expressing cell *in vivo*, e.g., a human carcinoma. The method comprises (i) administering to a subject a multi- or bispecific molecule of the invention or a fragment thereof, conjugated to a detectable marker; (ii) exposing the subject to a means for detecting said detectable marker to identify areas corresponding to a human
30 carcinoma. Protocols for *in vivo* diagnostic assays are provided in PCT/US88/01941, EP 0 365 997 and US 4,954,617.

35 The following invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Example 1: Production of Bispecific Antibody Comprising Humanized Antibodies Specific for an Fc Receptor and Anti-TAG72

5

Preparation of CC49xH22

Bispecific F(ab')₂ fragments were prepared according to modified techniques of Brennen and coworkers (Brennen *et al.* (1985) *Biotechniques* 4(5)). In brief, F(ab')₂ fragments of mAbs CC49 and H22 were made by pepsin digestion (Lamoyi, E. (1986)

- 10 *Methods Enzymol.* 121: 652-63; Lamoyi and Nisonoff, (1983) *J. Immunol. Methods* 56(2): 235-43). The F(ab')₂ fragments were then reduced with 0.5 mM DTT for 30 min. at 30°C. The reduced fragments were then treated with 5,5'-dithionitrobenzoic acid (DTNB) for 30 minutes at room temperature. The H22 Fab'-TNB derivative was purified by G25 gel filtration. MAb CC49 F(ab')₂ fragments were reduced with 15 mM
15 Mercaptoethylamine (MEA) for 30 min at 30°C and purified by G25 gel filtration. Equimolar amounts of H22 Fab'-TNB and CC49 Fab'-SH were mixed and incubated overnight at 4°C. The bispecific conjugate was purified from the uncoupled Fab' fragments by gel filtration chromatography (Superdex 200, Pharmacia, Piscataway, NJ).

20 *Protein Analysis*

Samples of CC49 F(ab')₂ and HCC49xH22 were run on a 4-15% tris-Glycine gel (BioRad, Hercules, California) at 5 ug/well. Gels were stained with Commassie Blue and analyzed on a Sun Sparcstation IPC densitometer (Millipore, Marlborough, Mass.).

25 *Flow Cytometry*

Kleb or U937 cell lines were washed with PBS containing 0.1% BSA (PBA), and added to 96-well plates at 3x10⁵/well. The plate was put on ice, and CC49 xFc_yRI diluted in PBA was added to wells in an equal volume. Where appropriate, an irrelevant antibody of similar species and isotype was used to determine non-specific binding.

- 30 Binding was allowed for 90 minutes at 4°C, followed by 3 washes with PBA. Phycoerytherin (PE) conjugated antihuman-IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was added at 1:50 in 50ml, and incubated at 4°C for 60 minutes. Following incubation with antihuman IgG-PE, the cells were washed 3 times with PBA and re-suspended in 400ml of 1% paraformaldehyde in PBS. Samples were
35 analyzed on Becton Dickinson FACScan.

- 33 -

Bifunctional Flow Cytometry Assay

Kleb, Jurkat, or CEM cells were washed with PBS containing 0.1% BSA (PBA) and added to 96 well plates at 3×10^5 /well. The plate was put on ice, and CC49 xH22diluted in PBA was added to wells in an equal volume. Binding was performed on 5 at 4°C for 90 min., followed by 3 washes with PBA. Cells bound with bispecific antibody were incubated with soluble FcγRI and human IgM Fc region (Brian Seed). The bound sFcγRI-m was then incubated with anti human IgM PE at 4° for 60 minutes. The cells were washed 3 times with PBA and re-suspended in 400 ul of 1% paraformaldehyde in PBS. Samples were analyzed on Becton Dickinson FACScan.

10

Antibody Dependent Cellular Cytotoxicity

Monocytes were purified from normal adult source leukocytes (Advanced Biotechnologies, Inc., Columbia, MD) by Ficoll Hypaque (Pharmacia, Piscataway, NJ) density gradient, followed by cold aggregation. Monocytes were cultured for 24-48 hr. 15 in teflon jars with M-SFM (macrophage-serum free medium M-SFM, Gibco, Grand Island, NY). 1000 U/ml IFN γ was added to up-regulate FcγRI expression. PMNs were purified from heparinized whole blood by lysis of red blood cells with 1.7M NH4Cl, 0.1 M KHCO₃, 1mM EDTA, after separation from mononuclear cells by Ficoll Hypaque density gradient centrifugation. PMNs were induced to express FcγRI with 50 ng/ml G- 20 CSF (kindly provided by Roland Repp, Erlangen, Germany) and 1000 U/ml IFN γ . On the day of the assay, monocytes or PMNs were washed and adjusted to 10^7 /ml or 2×10^7 /ml respectively. Target cells (Kleb, LS174T, Jurkat or CEM cells) were labeled with 100 mCi of ^{51}Cr for 1 hour and washed three times to remove excess ^{51}Cr and adjusted to a concentration of 10^5 /ml. Dilutions of antibodies (HCC49xH22, 25 HCC49Mab, H22F(ab')₂) were prepared in media and added with effector cells and targets cells to individual wells of microtiter plates in a volume of 50 ul each. Incubation times for ADCC ranged from 14 to 18 hours, then aliquots of supernatant were analyzed for radioactivity in a gamma counter. Cytotoxicity was calculated by the formula: % lysis = (experimental CPM -target leak CPM/ detergent lysis CPM - target 30 leak CPM) X 100%. Specific lysis is calculated by subtracting the % lysis without antibody from the % lysis with antibody. All samples were tested in triplicate.

Bispecific Antibody Conjugation

The bispecific antibody consisting of humanized antibody 22 (H22; produced by 35 a cell line having an ATCC accession number CRL11177) and humanized anti-TAG-72 antibody CC49 (HCC49), referred to herein as HCC49xH22, was prepared using the

- 34 -

chemical crosslinker DTNB, according to the method of Brennen *et al.* (1985) *Biotechniques* 4(5) described above. Using this chemical conjugation method, the H22 Fab'-TNB derivative was mixed with an equimolar amount of HCC49 Fab'-SH to produce a conjugated molecule linked via a thioether bond. Figure 1 depicts a schematic representation of the HCC49xH22 bispecific antibody. The conjugated molecules were separated from unreacted Fab' by a gel filtration step. Figure 2 shows an SDS PAGE analysis of HCC49xH22 demonstrating the relative purity with the expected molecular weight for F(ab')₂ bispecific antibody. As indicated above, lanes 1-4 show samples analyzed in reducing conditions, lanes 6-9 show samples analyzed in non-reducing conditions. Lanes 1 and 9 show the low molecular weight and high molecular weight standards, respectively; lanes 2 and 6 show a sample containing 5 µg of HCC49 X H22; lanes 3 and 7 show a sample containing 5 µg of H22 F(ab')₂; and, lanes 4 and 8 show a sample containing 5 µg of CC49 F(ab')₂.

15

Cell Binding

HCC49 X H22 bound specifically to Fc γ RI-expressing cells and to TAG-72-expressing cells as determined by flow cytometric analysis. Figures 3A-C show schematic representations comparing the binding of HCC49 X H22 to various human carcinoma cell lines. In particular, Figures 3A-C show the binding of HCC49 X H22 (c), H22 F(ab')₂ (b) and control to human endometrial carcinoma cells (Kleb cells; Fig. 3A); acute human T cell leukemia cells (Jurkat cells; Fig. 3B) and human colon carcinoma (LS174T; Fig. 3C).

Figures 4A-B show the saturable binding curves of HCC49 X H22 conjugates to Kleb cells (4A) and U-937 cells. To perform these experiments, 3x10⁵ Kleb cells or U-937 cells per well in a 96 well plate were stained with HCC49xH22 for 90 minutes at 4° C and detected with mouse anti-humanIgG-PE. To exclusively measure bispecific antibody binding activity, anti-humanIgG-PE directed to the nonbinding arm of HCC49 X H22 was used for detection. Figure 4A demonstrates that HCC49xH22 binds with high affinity to human carcinoma cell lines known to overexpress TAG-72, namely Kleb cells, for example, LS174T cells and Jurkat cells. As indicated, HCC49xH22 bound to Kleb cells at concentrations as low as 0.1 ug/ml. Figure 4A further provides a comparison of the binding of HCC49 X H22 (filled circles) and CC49 monoclonal antibody (empty circles) to Kleb cells. which indicates that the bispecific conjugate retains similar affinities to TAG-72 as the CC49 monoclonal antibody. Figure 4B

- 35 -

demonstrates binding of HCC49xH22 to the Fc γ RI-expressing U937 cells, which indicates that the H22 portion is capable of engaging Fc γ RI.

The bifunctional nature of HCC49xH22 was further demonstrated by the simultaneous binding of the bispecific antibody to soluble Fc γ RI and TAG-72-expressing cells, Kleb or Jurkat cells. Figures 5A-B show the simultaneous binding of HCC49 x H22 (filled circles) or control anti-EGF x anti-Fc γ RI (solid circles) to Kleb cells and soluble Fc γ RI. Saturable binding to the carcinoma or the leukemia cells was detected using HCC49 X H22, but not with the control anti-EGF x anti-Fc γ RI bispecific antibody. To perform the experiments in Figure 5A, 3×10^5 Kleb cells/well were stained with HCC49xH22 or anti-EGFr x Fc γ R1 followed by soluble Fc γ R1 and detected with anti-humanIg MPE. Figure 5B shows the simultaneous binding of HCC49 x H22 to soluble Fc γ RI and Kleb cells (solid circles), Jurkat cells (empty circles) or CEM cells (filled inverted triangle). To perform the experiments shown in Figure 5B, 3×10^5 Kleb, Jurkat or CEM cells/well were stained with CC49xFc γ R1 or anti-EGFr x Fc γ R1 followed by soluble Fc γ R1 and detected with antihumanIgMPE. Saturable binding was detected using HCC49 x H22 in Kleb and Jurkat, but not with the anti-TAG-72-negative CEM cell line. Thus, the detected binding activity of HCC49xH22 seems to result from bispecific antibody binding.

20 *Antibody Dependent Cellular Cytotoxicity*

The cytotoxic activity of the bispecific antibody was examined using Fc γ RI-expressing monocyte and PMN effector cells. Although monocytes express significant levels of Fc γ RI constitutively, culturing these cells with IFN- γ results in up-regulation of Fc γ RI (Perussia, B. *et al.* (1983) *J. Exp. Med.* 158: 1092-1113; Guyre, P.M. (1983) *J. Clin. Invest.* 72: 393-97). IFN- γ -treated monocytes mediated specific lysis of TAG-72-expressing target cells (Kleb cells, LS174T cells and Jurkat cells) in a bispecific antibody-dependent fashion that saturated approximately at 100 ng/ml (Figures 6A-B). The relative percentage cell killing of the cell lines in these assays correlates with the binding of HCC49 x H22 to the cells as demonstrated by the flow cytometry assays.

30 More specifically, Figure 6A shows a comparison of dose response curves demonstrating induction of ADCC of Kleb cells (filled circles) or LS174T cells (empty circles) with respect to the concentration of HCC49 x H22 (μ g/ml). Figure 6B shows a comparison of dose response curves demonstrating induction of ADCC of Kleb cells (filled circles) or Jurkat cells (empty circles) with respect to the concentration of HCC49 X H22 (μ g/ml). To perform, the experiments shown in Figures 6A-B, monocytes were purified from leukopak and plated at 5×10^5 /well with 5000 targets/well and appropriate

- 36 -

concentration of CC49xFc γ R1 antibody. Plates were incubated overnight and supernates were harvested and read on a gamma counter. % lysis = (experimental CPM-target leak CPM/Mas lysis CPM) x 100 % specific lysis = spontaneous lysis-lysis with antibody).

5 Figure 7 demonstrates the induction of ADCC of TAG-72 overexpressing Kleb cells using HCC49 x H22 using γ IFN and GCSF treated PMNs as effectors. Although Fc γ RI expression on circulating PMN cells is very low, up-regulation with G-CSF and/or IFN- γ enables PMN cells to mediate efficient ADCC through Fc γ RI. PMN cells were cultured with cytokines prior to use as effector cells for ADCC. Figure 7 shows a
10 dose response curve demonstrating induction of ADCC of Kleb cells with respect to the concentration of HCC49 X H22 (filled circles) or CC49 monoclonal antibody (empty circles).

15 In sum, the results presented herein indicate that HCC49xH22 binds with high affinity to both TAG 72 and Fc γ R1-expressing cells. More importantly, these results demonstrate that HCC49xH22 effectively mediates antibody dependent cell cytotoxicity against TAG-72 expressing cells when using monocytes and activated PMNs as effector cells. Therefore, these results support the conclusion that the fully humanized bispecific antibody, HCC49 x H22, may be a promising therapy for treatment of TAG-72 expressing cancers.

20

Equivalents

25 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

- 37 -

We claim:

1. A bispecific molecule comprising a first binding specificity for an Fc γ receptor (Fc γ R) and a second binding specificity for the tumor associated glycoprotein 72.
5
2. The bispecific molecule of claim 1 which binds to the Fc γ receptor at a site which is not blocked by endogenous immunoglobulin G.
3. The bispecific molecule of claim 1, wherein said first binding specificity
10 recognizes an Fc γ receptor selected from the group consisting of Fc γ RI, Fc γ RII and Fc γ RIII.
4. The bispecific molecule of claim 1, wherein said first binding specificity
recognizes Fc γ RI.
15
5. The bispecific molecule of claim 1 which is a bispecific antibody.
6. The bispecific molecule of claim 1 which comprises at least one humanized
antibody or fragment thereof.
20
7. The bispecific molecule of claim 1, wherein said first binding specificity
comprises a humanized anti-Fc γ RI antibody or fragment thereof.
8. The bispecific molecule of claim 7, wherein said humanized anti-Fc γ RI antibody
25 is the humanized antibody 22 (H22) or fragment thereof produced by a cell line having
an ATCC accession number CRL11177.
9. The bispecific molecule of claim 1, wherein said second binding specificity is
provided by a humanized anti-tumor associated glycoprotein 72 antibody or fragment
30 thereof.
10. The bispecific molecule of claim 1, wherein said humanized anti-tumor
associated glycoprotein 72 antibody is the humanized antibody CC49 or fragment
thereof.

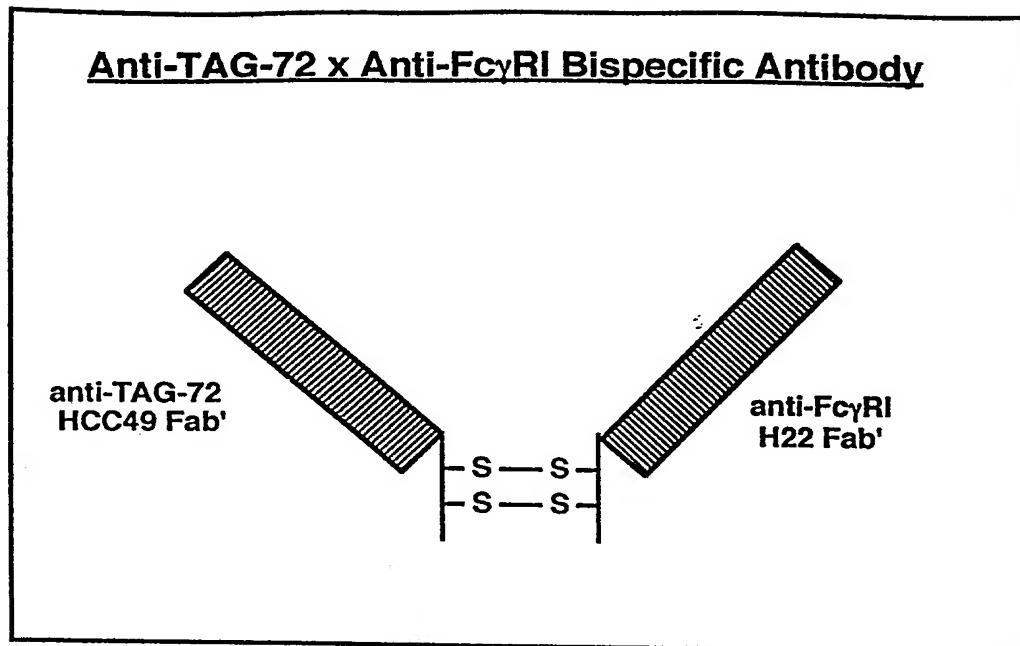
- 38 -

11. A bispecific molecule comprising a humanized antibody 22 or a fragment thereof produced by a cell line having an ATCC accession number CRL11177, and a humanized anti-tumor associated glycoprotein 72 antibody (HCC49) or a fragment thereof.
- 5 12. A composition comprising comprising a bispecific molecule of claim 1, and a pharmaceutically acceptable carrier.
- 10 13. A target-specific effector cell comprising (a) an effector cell expressing an Fc γ receptor, and (b) a bispecific molecule bound to the Fc γ receptor, said bispecific molecule comprising a first binding specificity for an Fc γ receptor (Fc γ R) and a second binding specificity for the tumor associated glycoprotein 72.
- 15 14. The target-specific effector cell of claim 13, wherein said effector cell is selected from the group consisting of a lymphocyte, a killer cell, a natural killer cell, a macrophage, a monocyte, an eosinophil, a neutrophil, a polymorphonuclear cell, a granulocyte, a mast cell, and a basophil.
- 20 15. A method of triggering at least one Fc γ -receptor mediated effector cell function comprising contacting an effector cell expressing an Fc γ receptor with a bispecific molecule comprising a binding specificity for an Fc receptor and a second binding specificity for a tumor associated antigen 72.
- 25 16. The method of claim 15, wherein said at least one Fc γ -receptor mediated effector cell function is selected from the group consisting of antibody dependent cellular toxicity (ADCC), phagocytosis of a target cell, cytokine release and generation of superoxide anion.
- 30 17. The method of claim 16, wherein the target cell is a tumor associated glycoprotein 72-expressing cell.
18. The method of claim 17, wherein the tumor associated glycoprotein 72-expressing cell is selected from the group consisting of a tumor cell and an endometrial cell.
- 35 19. The method of claim 18, wherein the tumor cell is selected from the group consisting of a colon, breast, prostate, ovarian and endometrial cancer cell.

- 39 -

20. The method of claim 15, wherein the effector cell is selected from the group consisting of a lymphocyte, a killer cell, a natural killer cell, a macrophage, a monocyte, an eosinophil, a neutrophil, a polymorphonuclear cell, a granulocyte, a mast cell, and a
5 basophil.
21. A method of treating or preventing a disorder characterized by abnormal functioning of a tumor associated glycoprotein 72-expressing cell, comprising administering to a subject a bispecific molecule comprising a binding specificity for an
10 Fc receptor and a second binding specificity for a tumor associated antigen 72 in an amount effective to inhibit the activity of the tumor associated glycoprotein 72-expressing cells.
22. The method of claim 21, wherein the tumor associated glycoprotein 72-expressing cell is a tumor cell or an endometrial cell.
15

1/7

**FIGURE 1**

2/7

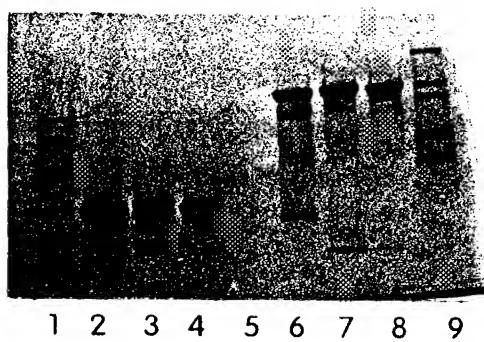


Fig. 2

3/7

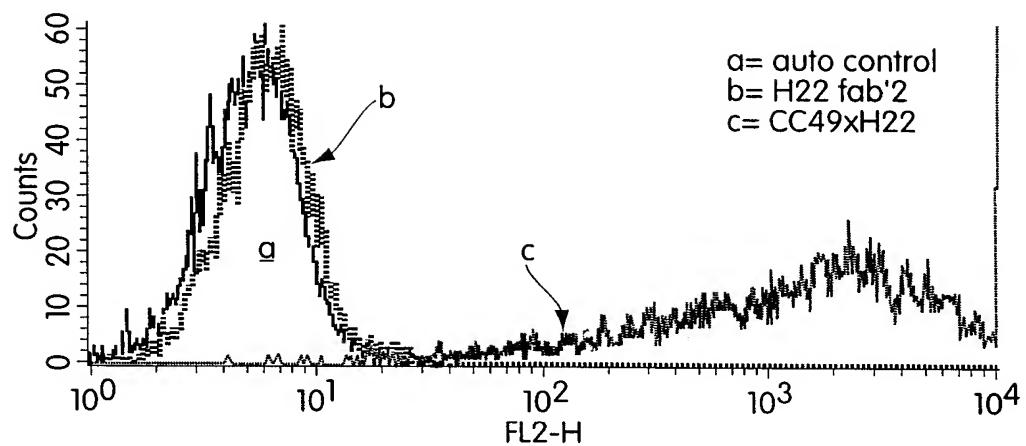


Fig. 3A

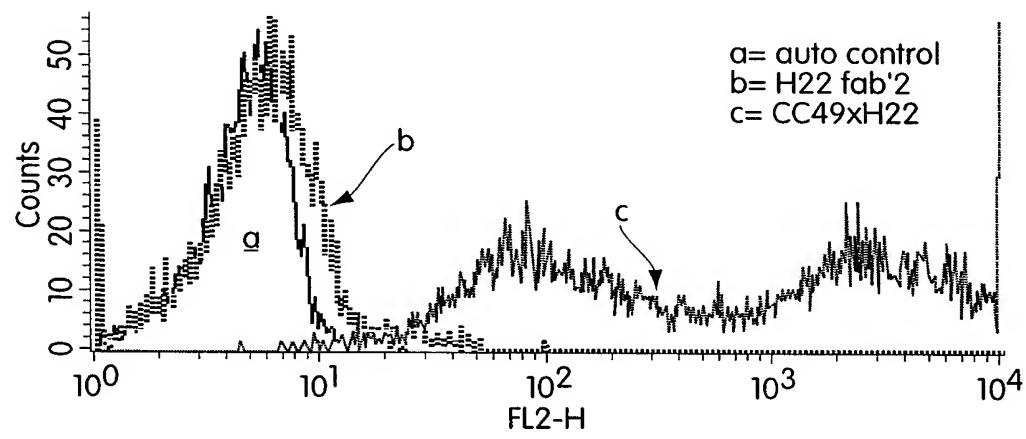


Fig. 3B

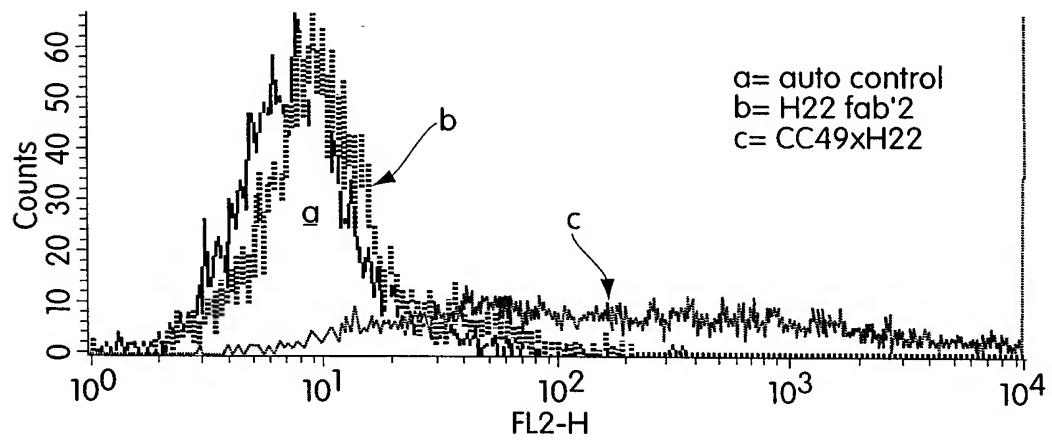


Fig. 3C

4/7

Flow cytometry assays

FIGURE 4A

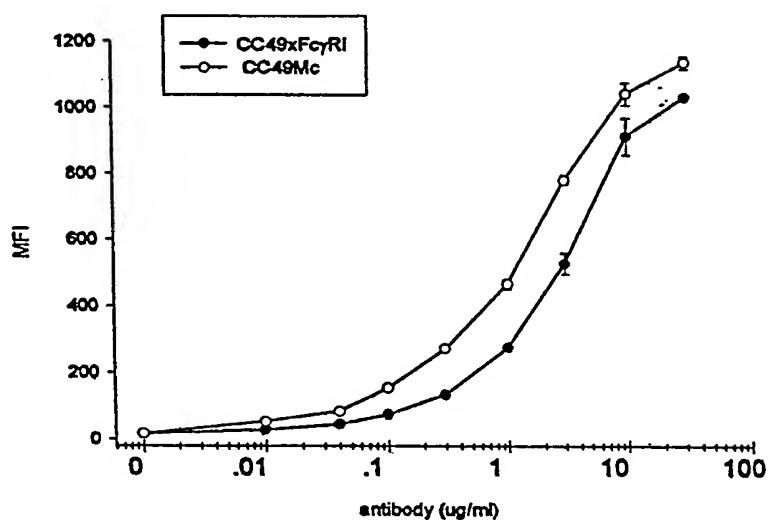
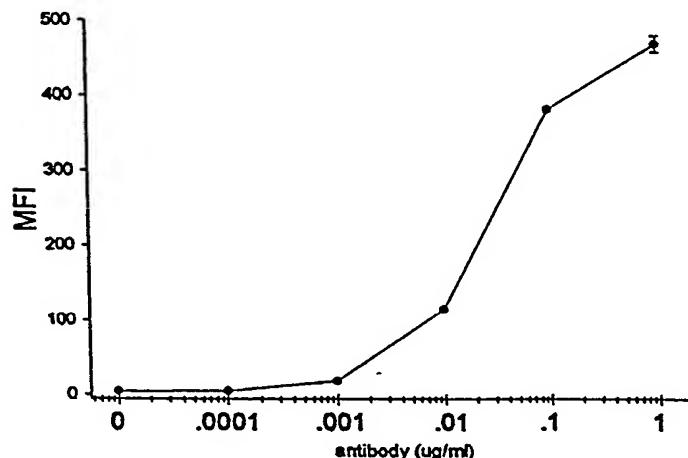


FIGURE 4B

Binding CC49 x FcgRI to U937 cells as measured by flow cytometry



5/7

Bifunctional flow cytometry assays

FIGURE 5A

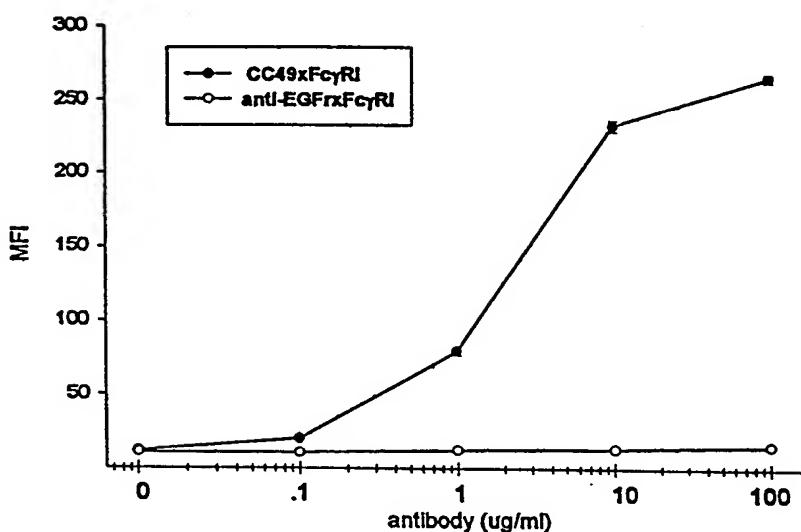
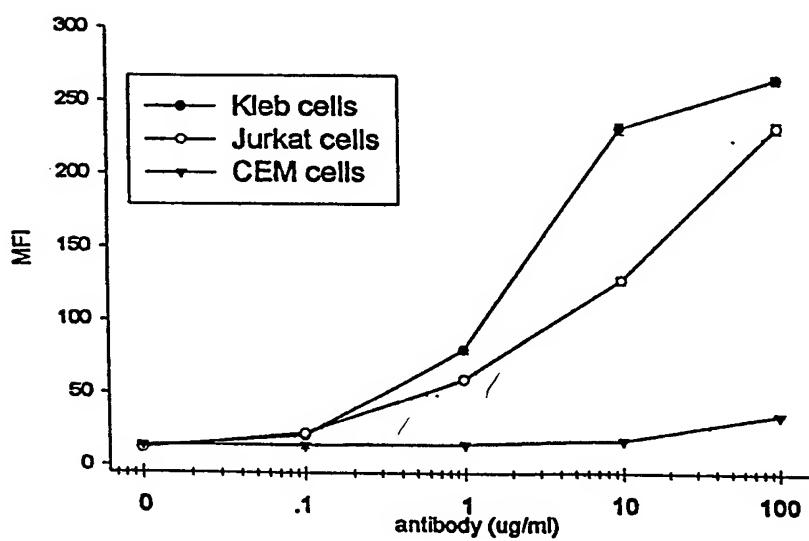
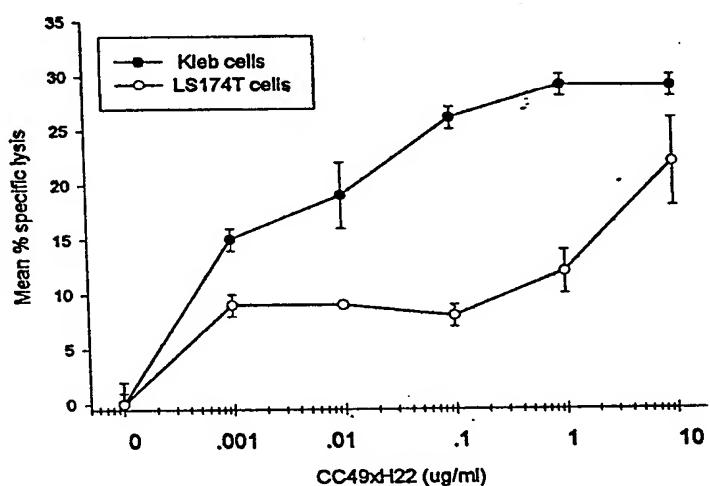
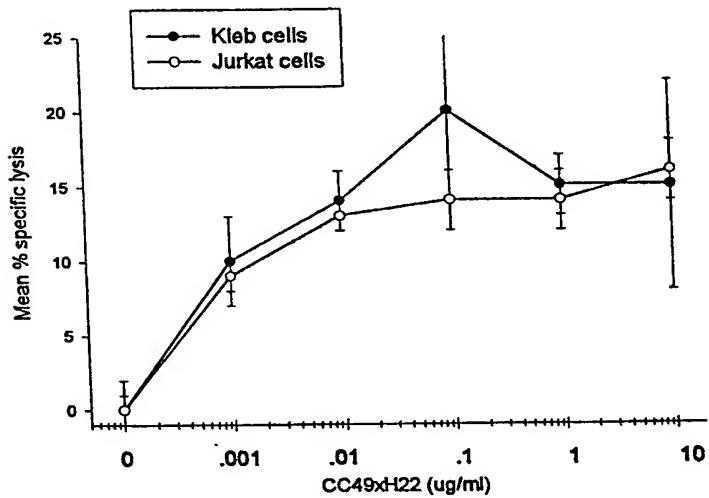


FIGURE 5B



6/7

**ADCC with CC49xFc γ RI using γ INF treated monocytes as effectors
in overnight assay****FIGURE 6A****FIGURE 6B**

7/7

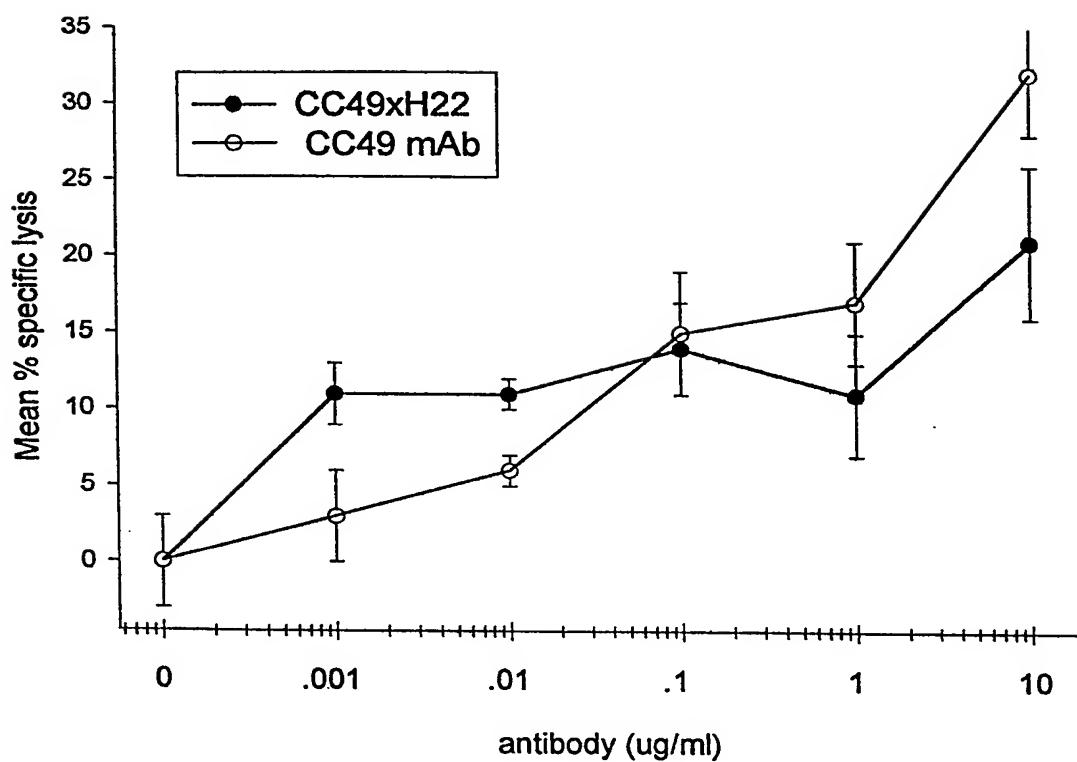


FIGURE 7

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18428

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 6	C07K16/28	C07K16/30	C07K16/46	C12N5/08	A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUSSONIELLO C. ET AL.: "In vitro antitumor activity of anti-TAG-72xanti-FcgRI bispecific antibody" AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 38, 12 - 16 April 1997, page 27 XP002066247 see the whole document	1-8, 13-22
Y	---	9-12
Y	SLAVIN-CHIORINI DC. ET AL.: "Biological properties of chimeric domain-deleted anticarcinoma immunoglobulins" CANCER RESEARCH, vol. 55, no. suppl. 1 December 1995, pages 5957-5967, XP002066248 cited in the application see abstract	9-12
	---	-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

29 May 1998

Date of mailing of the international search report

15.07.1998

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Covone, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 97/18428

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>POSEY J. ET AL.: "Pilot trial of MDX-H210 and gm-CSF for patients with advanced erbB-2 positive malignancies" AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol. 37, 20 - 24 April 1996, page 165 XP002066249 see the whole document</p> <p>---</p>	1-22
Y	<p>WO 93 12231 A (DOW CHEMICAL AUSTRALIA) 24 June 1993 cited in the application see abstract see page 33, line 1 - line 6</p> <p>-----</p>	1-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/18428

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15-20 (all partially, as far as they concern an in vivo treatment) and 21, 22 (both completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/18428

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9312231	A	24-06-1993	CA 2121041 A	24-06-1993
